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Review

Nucleolus and rRNA Gene Chromatin in Early Embryo Development

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The nucleolus is the largest substructure in the nucleus and forms around the nucleolar organizer regions (NORs), which comprise hundreds of rRNA genes. Recent evidence highlights further functions of the nucleolus that go beyond ribosome biogenesis. Data indicate that the nucleolus acts as a compartment for the location and regulation of repressive genomic domains and, together with the nuclear lamina, represents the hub for the organization of the inactive heterochromatin. In this review, we discuss recent findings that have revealed how nucleolar structure and rRNA gene chromatin states are regulated during early mammalian development and their contribution to the higher-order spatial organization of the genome.

Highlights

The nucleolus together with the nuclear lamina is the hub for the organization of the inactive heterochromatin.

The structure of the nucleoli and chromatin states of rRNA genes undergo major changes during gametogenesis and early mammalian development.

The nucleolus and the chromatin composition of rRNA genes contribute to the higher-order spatial organization of the genome.

The Multifunctional Nucleolus: From Ribosome Producer to Organizer of Genome Architecture

The **nucleolus** (see Glossary) is the largest subnuclear compartment of the cell and forms around regions of chromosomes containing stretches of tandem repetitive rRNA genes, known as **nucleolar organizer regions (NORs)**. The nucleolus is responsible for the production of ribosomes, a highly regulated process that is essential for growth and development. **Ribosome biogenesis** is initiated in the nucleolus by RNA polymerase I (Pol I), which, together with a dedicated set of basal transcription factors, such as TIF1A, the TBP-TAFI complex SL1, and the DNA architectural upstream binding factor (UBF) [1,2], transcribes hundreds of rRNA genes to generate 45S/47S pre-rRNA in mammalian cells (Figure 1A). This rRNA precursor is chemically modified and processed to form 28S, 18S, and 5.8S rRNAs, which are then assembled with ribosomal proteins and 5S rRNA and exported from the nucleus to give rise to active ribosomes in the cytoplasm [3].

Increasing evidence suggests that the function of the nucleolus and rRNA genes goes beyond ribosome biogenesis. One aspect that has received significant interest over the past decade is the link between the nucleolus and rRNA genes with the organization of genome architecture. Clustering of **heterochromatin** at nucleoli is a phenomenon that occurs in all cells. Furthermore, regions located close to the nucleolus (nucleolar-associated domains, NADs) have low gene densities, low transcriptional levels, and repressive histone modifications [4–8]. Since similar heterochromatic and repressive regions are also located to the nuclear lamina, the nucleolus, together with the nuclear lamina, can be considered the hub for the organization of the inactive chromatin in the cell [8–10].

In this review, we address the role of the nucleolus as a subnuclear compartment for the organization of heterochromatin. We discuss recent findings that have revealed how nucleolar structure and rRNA gene chromatin states are regulated during **gametogenesis** and early mammalian development, and their contribution in the higher-order spatial organization of the genome.

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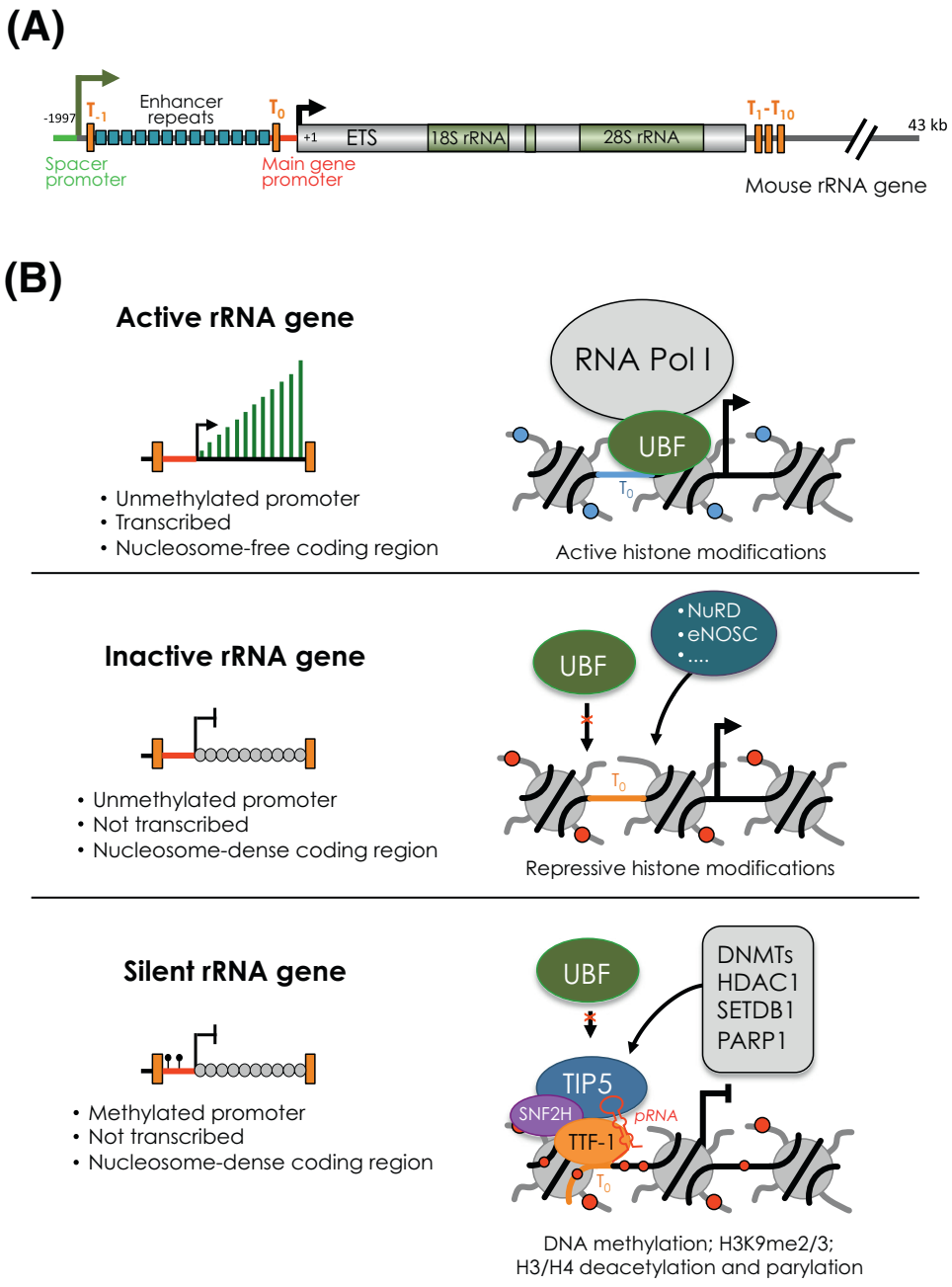

Figure 1. The Three Major Classes of rRNA Gene. (A) Structural organization of mouse rRNA genes. The sites of transcription initiation of the 45S pre-rRNA from the main gene promoter and intergenic spacer (IGS)-rRNA transcripts from the spacer promoter are indicated by arrows. Terminator elements downstream of the spacer promoter (T_{-1}), upstream of the main gene promoter (T_0), and downstream of the coding regions (T_1–T_{10}) are marked by orange bars. The repeats comprising the enhancer (13 according to the sequence from Genbank accession number BK000964) are shown as blue bars. (B) Description of active, inactive, and silent rRNA genes based on transcription, chromatin, and epigenetic features, and factors regulating their state. The composition of the main rRNA gene promoter is described. The binding of upstream binding factor (UBF) and nucleolar remodeling complex (NoRC) (TIP5 and SNF2H) define active and silent rRNA

(Figure legend continued at the bottom of the next page.)

Glossary

Centromeric heterochromatin: regions of the inner centromere that comprise minor satellite repeats in mouse cells.

Chromocenters: structures visible in mouse interphase cells, containing pericentromeric and centromeric sequences of several chromosomes that cluster together.

Euchromatin: chromatin regions that are less condensed, gene-rich, and more accessible to transcription. Euchromatin is typically enriched in active histone marks.

Fertilization: process involving the fusion of gametes to form a new organism of the same species. In animals, this process involves a sperm fusing with an oocyte, thereby forming the zygote.

Gametogenesis: production of haploid sex cells in mammals, each carrying half of the genetic complement of the parents. In this process, diploid gametes undergo meiosis and differentiation. Gametes of male and female reproductive systems are known as sperms and oocytes, respectively. The corresponding processes are called spermatogenesis and oogenesis.

Heterochromatin: chromatin regions that are highly condensed, gene-poor, and transcriptionally silent. Heterochromatin is characterized by repressive histone marks and DNA methylation in organisms showing this modification. Regions that contain a high density of repetitive DNA elements, such as clusters of satellites and transposons, are the main targets for heterochromatin formation.

Nucleolar organizer regions (NORs): chromosomal regions containing rRNA genes. In humans and apes, rRNA genes are located between the short arm and the satellite body of acrocentric chromosomes 13, 14, 15, 21, and 22. In mouse cells, rRNA repeats are within the centromeric regions of chromosomes 12, 15, 16, 18, and 19.

Nucleolus: largest subnuclear compartment of the cell, where ribosome biogenesis takes place. The nucleolus forms around regions of chromosomes containing stretches of rRNA gene repeats, known as NORs. It is a membraneless compartment. Nucleoli of somatic cells show a peculiar tripartite architecture: the FC, DFC, and GC.

Nucleolus and rRNA Genes in Somatic Cells

In somatic cells, the nucleolus is a membraneless compartment with a peculiar tripartite architecture: the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). These nucleolar subcompartments represent distinct, coexisting liquid phases [11]. These structures facilitate the progressive stages of ribosome biogenesis, such as rRNA transcription, which occurs at the boundary between FC and DFC; rRNA processing, which is located in the DFC; and ribosome assembly, which takes place in the GC [12,13].

Transcription of rRNA genes in the nucleoli produces most of the RNAs in the cell. This large rRNA synthesis is mainly due to the high transcription rate [approximately one Pol I every 130 nucleotides (nt)] [14–16]) and the presence of multiple copies of rRNA genes (in mammalian cells, ~200 per haploid genome). However, in somatic cells, even under high cell proliferative conditions, not all rRNA genes within a cell are competent for transcription [17].

rRNA genes can be subdivided into three major classes according to their transcription and chromatin states: silent, inactive, and active genes [18] (Figure 1B). In mammalian cells, DNA methylation at the promoter distinguishes silent rRNA genes from the rest of the repeats [19]. Promoter methylation abrogates the formation of the Pol I pre-initiation complex by impairing the binding of UBF [19]. Silent rRNA genes display constitutive heterochromatic features and associate with repressive histone marks [20,21]. The responsible factor for the establishment of silent rRNA genes is the nucleolar remodeling complex (NoRC), which comprises TTF-1 interacting protein 5 (TIP5) and SNF2H, a member of the ISWI subfamily and catalytic subunit of several chromatin-remodeling complexes [20–22] (Figure 1B). NoRC is recruited to rRNA genes through its association with the long noncoding (lnc)RNA pRNA; pRNA mediates the interaction with TTF-1, which binds the terminator DNA element T_0 located in the rRNA gene promoter [23–25]. pRNA is generated by the RNA helicase DHX9-mediated processing of the intergenic spacer (IGS)-rRNA, a transcript that originates from the rRNA spacer promoter and which is located 2 kb upstream of the main gene promoter in mouse cells [24,26] (Figure 1A). NoRC establishes silent rRNA genes through the recruitment of DNA methyltransferases (DNMTs) and histone modifier enzymes, such as histone deacetylase 1 (HDAC1), thereby establishing heterochromatic and repressive structures [20,21,27]. rRNA genes lacking CpG methylation at the promoter can be classified as active or inactive repeats. Active rRNA genes are transcriptionally active, associate with Pol I and UBF, contain histone modifications linked to **euchromatin**, and have a coding region that is nucleosome-free (Figure 1B). By contrast, inactive genes do not transcribe, do not interact with UBF, and display nucleosome-packed chromatin, as in the case of silent rRNA genes. The structure of inactive rRNA genes can be mediated by the energy-dependent nucleolar silencing complex (eNoSC) and the nucleosome remodeling and deacetylase (NuRD) complex [28,29]. UBF is the key factor for the establishment of active rRNA genes [30,31]. Depletion of UBF switches rRNA genes from an active to an inactive state. This process is reversible because the re-expression of UBF re-establishes active rRNA copies [30].

Nucleolus precursor body (NPB): an atypical nucleolus that is formed in zygotes and persists until the eight-cell stage of early development. NPBs are transcriptionally inactive and morphologically similar to the oocyte NPB, lacking the tripartite configuration of a typical somatic nucleolus.
Nucleolus-like body (NLB): an atypical nucleolus that is formed in fully grown GV oocytes. NLB structure is compact and morphologically different from nucleoli in somatic cells.
Pericentromeric heterochromatin: highly condensed regions of the genome that comprise major satellites, which flank the centric domain, which consists of 'minor' satellite repeats.
Pluripotency: the state of a cell within the early mammalian embryo that has the capacity to generate all somatic lineages and the germline. Pluripotency is confined to the preimplantation epiblast. *In vitro*, pluripotency can be maintained indefinitely through derivation of ESC lines.
Ribosome biogenesis: process that requires the coordinated activity of all three RNA polymerases and the orchestrated work of many (>200) transiently associated ribosome assembly factors to produce ribosomes.
Totipotency: state of a cell that can give rise to all the extraembryonic tissues and all tissues of the body and the germline. Establishment of totipotency is initiated upon fertilization with the formation of the zygote.

genes, respectively. Inactive rRNA genes are nontranscribed repeats that lack promoter DNA methylation, are nucleosome-packed at the coding region, and are not bound by either UBF or NoRC [20,30]. The structure of inactive genes can be mediated by energy-dependent nucleolar silencing complex (eNoSC), nucleosome remodeling and deacetylase (NuRD), or other as yet unknown regulators [28,29]. Establishment of silent rRNA genes is described. NoRC is recruited to the promoter through the interaction with TTF-1 mediated by the long noncoding (lnc)RNA pRNA [23]. Subsequently, NoRC recruits factors that establish epigenetic silencing, such as DNA methyltransferase (DNMTs), histone deacetylase (HDAC1), histone methyltransferases (SETDB1), and poly(ADP-ribose) polymerase 1 (PARP1) [20,21,91,92]. Abbreviations: EST, external transcribed spacer; RNA Pol 1, RNA polymerase 1.

The structure and composition of nucleoli and rRNA genes observed in somatic cells undergo drastic changes in oogenesis and early development. These alterations are thought to have important roles in cell reprogramming. Here, we describe and discuss the changes that occur at the nucleolus and in rRNA genes and their functional role during early development.

Nucleolus Structures and rRNA Genes in Gametogenesis

The process of gametogenesis, **fertilization**, and preimplantation developments (Box 1) are characterized by tightly coordinated molecular events that are crucial to ensure successful embryonic development. These changes include histone and DNA modifications, higher-order chromatin organization, genome compartmentalization, and nucleolus structure.

In mammals, oogenesis and spermatogenesis occur at very different times during development and achieve different endpoints. In males, meiosis is initiated at the onset of puberty [32]. In many metazoans, male germ cells undergo an extensive chromatin-remodeling process during their final differentiation into sperm, during which genomic DNA becomes transcriptionally silent, highly methylated, and packaged with protamines into a highly condensed configuration [33].

Oogenesis is initiated in the fetus well before birth, forming a finite number of gametes that are used periodically over a defined reproductive lifetime. The mammalian oocyte nucleus or germinal vesicle (GV) exhibits a unique chromatin configuration that is subject to dynamic modifications during oogenesis (Figure 2, Key Figure). Upon entering the growth phase, oocytes synthesize large amounts of material, including ribosomes, and markedly enlarge in diameter. When oocytes reach their full size, they acquire the competence to disassemble their nuclei and undergo meiotic maturation. However, not all oocytes have a full developmental potential when fertilized [34]. During the very final growth phase, oocytes acquire two main types of nuclear organization: the surrounded nucleolus (SN) type and the nonsurrounded nucleolus (NSN) type. The SN type is transcriptionally inactive, whereas the NSN type is transcriptionally active [35,36]. Although both types of oocyte are able to resume meiosis and undergo fertilization, they do not have the same developmental competence [37–39]. Following fertilization, NSN-derived embryos stop developing at the two-cell stage, whereas SN-derived embryos pursue development until the blastocyst stage and reach full-term [37]. Remarkably, during the very final phases of the growth period, SN oocytes gradually shut down both RNA Pol I and II activities [40] and the tripartite structure of their nucleoli undergoes gradual changes to transform into atypical nucleoli termed '**nucleolus-like bodies' (NLB)** (Figure 2). At the morphological level, NLBs comprise tightly packed fibers of 6–10 nm [41]. Importantly, SN oocytes display a dense chromatin ring around the NLB, whereas the chromatin in NSN is uncondensed. **Pericentromeric heterochromatin** in

Box 1. Preimplantation Developmental Stages

A new live multicellular organism develops from a single zygote that was created by the fusion of two specialized and highly differentiated cells, the oocyte and the sperm, a process termed 'fertilization'. Early mammalian embryo develops through several recognizable cellular events, known as preimplantation embryogenesis. Preimplantation development includes the period from zygote formation (one-cell embryo) until invasion of the uterine epithelium by the blastocyst occurs [89]. After fertilization, a one-cell embryo (zygote) is formed and undergoes three rounds of cleavage division, resulting in a two-cell, four-cell, and eight-cell stage embryo, respectively, together known as blastomeres. The following formation of the morula represents the earliest point where blastomeres have differential spatial positioning. Over subsequent asymmetric cleavage divisions, the embryo develops further into the blastocyst, containing two specific cell types: trophectoderm (TE) and inner cell mass (ICM) cells. At the late blastocyst stage, the ICM consolidates to establish the epiblast (EPI) and the primitive endoderm (PE) lineages. At this point, the epiblast cells enter the developmental 'ground state,' the origin of all future embryonic lineages [90]. During the final step of preimplantation development, the blastocyst is ready to attach to the uterus, and further development takes place.

Key Figure

The Nucleolar Cycle in the Germ Line and during Early Development

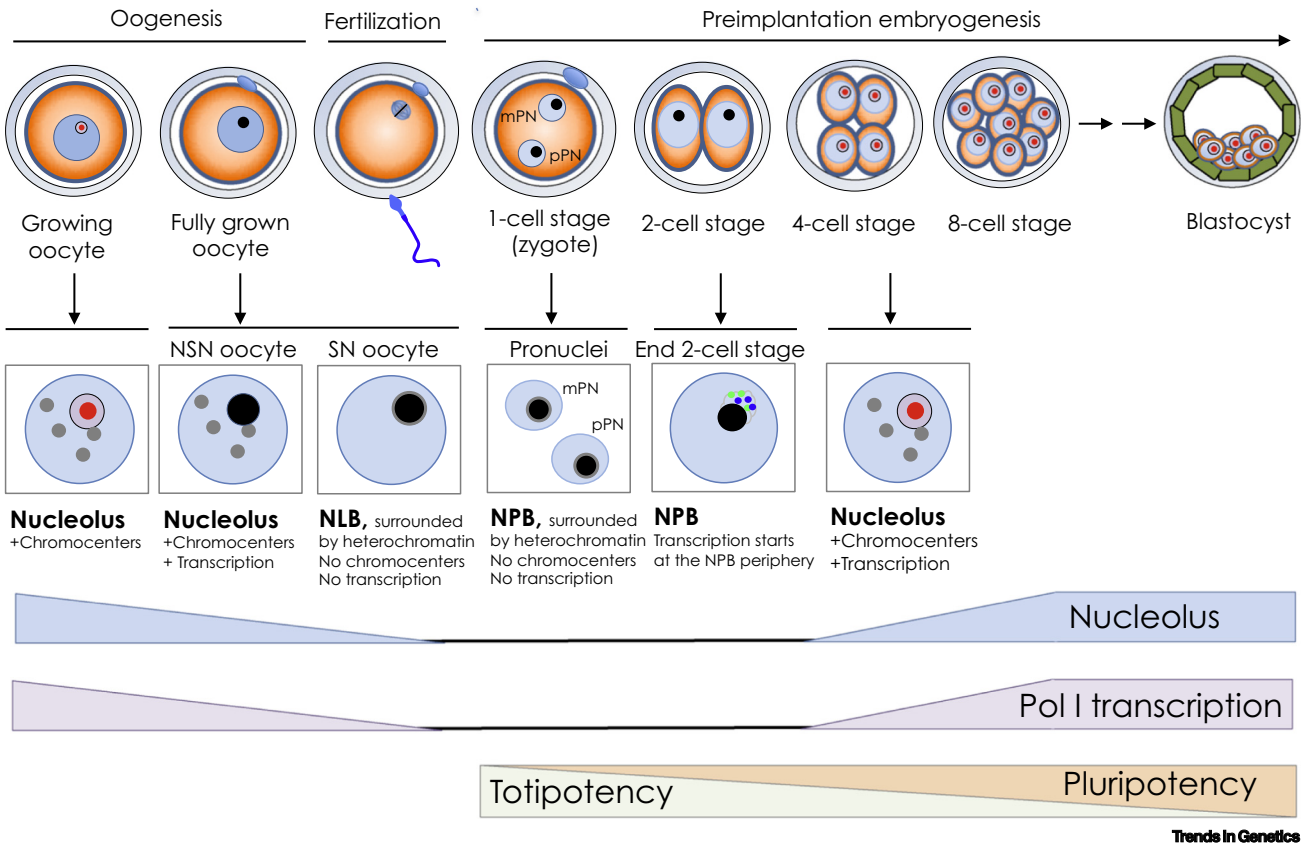

Figure 2. Illustration of mouse oogenesis and early developmental timing. The nucleoli of growing oocytes in mammals comprise fibrillar centers (red), dense fibrillar components, and granular components (purple). At the end of the growth phase, the nucleolus is transformed into the nucleolus-like body (NLB, black), which lacks the tripartite structure. Oocytes acquire two main types of nuclear organization: the surrounded nucleolus (SN) type and nonsurrounded nucleolus (NSN) type. The SN type is transcriptionally inactive and the NLB is surrounded by heterochromatin in a ring-like shape (gray lane). The NSN type is transcriptionally active and displays aggregation of heterochromatin in chromocenters (gray circles). During the final stage of oocyte maturation, the nucleoli (NLBs) disappear and reappear, upon fertilization, in the pronuclei of the zygote in the form of nucleolus precursor bodies (NPB, black). At the end of the two-cell stage, transcription is initiated at the surface of NPB where UBF (green) and Nopp140 (blue) localize. From the four-cell stage onwards, the NPB gradually transforms into somatic nucleoli that display the tripartite structure. Zygotic remnants of NPBs persist up to the eight-cell stage. Timing of rRNA gene transcription, timing of the development of the nucleolus, and the transition from totipotency to pluripotency are shown.

NSN oocytes aggregates within **chromocenters**, whereas, in SN oocytes, it forms a discontinuous ring around the NLB [42]. During the following phase of oogenesis, the full-size grown oocyte undergoes meiotic maturation, with the breakdown of the nuclear envelope and consequent dispersion of NLBs into the cytoplasm [43]. Although most of the nuclear material is retained by the eggs, at Metaphase II, transcripts of several nucleolar proteins, such as UBF and fibrillarin, are largely degraded and decline even further after fertilization, reaching the lowest levels at the two-cell stage, when embryonic genome activation (EGA) occurs in the mouse [44]. The appearance of paternal transcripts at this stage led to the suggestion that the maternal transcripts are eliminated, and these nucleolar factors are produced by the embryo.

Nucleolus and rRNA Genes during Early Embryo Development

Fertilization occurs when the two highly differentiated cells, the oocyte and the sperm, fuse to form the zygote. Given that both parental genomes are transcriptionally silent, the initial development of the embryo exclusively depends on maternally inherited RNAs and proteins [45]. EGA is established in two phases: a 'minor activation', which occurs at the one-cell stage in most species [46], followed by a second 'major activation' that in mouse takes place during the two-cell stage [47]. rRNA gene transcription starts only at the end of the two-cell stage and is required for further embryonic development. Embryos lacking RNA polymerase 1-2 (RPO1-2) or UBF arrest development before the morula stage [48,49]. Furthermore, pharmacological inhibition of rRNA gene transcription using CX-5461 [50] induced developmental delay and arrest at the four-cell stage [51].

Upon fertilization, the NLBs that disappeared during oocyte meiotic maturation reappear in both maternal and paternal pronuclei of zygotes in the form of **nucleolus precursor bodies (NPBs)**, which persist throughout the first four cell cycles [52] (Figure 2). Later during development, during the transition from the 16-cell to the morula stage, NPBs gradually disappear and are replaced by somatic-type nucleoli with classic tripartite structure. In contrast to NLBs, NPBs are significantly impoverished for RNA and detectable amounts of RNA appear on the NPB surface only after resumption of rRNA gene transcription [53]. Accordingly, during the late two-cell and four-cell stages, UBF and Nopp140, two nucleolar proteins that are associated with FC and DFC of the nucleolus, localize around the periphery of the NPBs [51]. These results also suggest that active rRNA genes, which are bound by UBF [19,20], are formed at the outer surface of the NPB. Starting at the eight-cell stage, NPBs diminish in size, disappearing by the late 16-cell stage, with the emergence of intermingled fibrillar and granular compartments and UBF positioning in the center of the former NPB [51]. Since nucleoli and activation of rRNA genes occurs at the periphery of NPBs, it has been suggested that NPBs are the building blocks of the future somatic nucleoli. Data also suggest that the re-establishment of nucleoli during early embryo development is tightly linked to transcription. Inactivation of Pol I with the inhibitor CX-5461 led to a reorganization of Nopp140 and UBF, which formed nucleolar caps at both the two- and four-cell stages [51]. These caps corresponded to those observed in SN oocytes and in interphase cells treated with inhibitors of Pol I transcription. Similar structures were also observed in UBF-null embryos, where NPBs were also either no longer apparent or strongly perturbed [48].

Increasing evidence suggests that the role of NPB goes beyond the simple transmission of nucleolar components from female germ cells to the embryo and could participate in the remodeling of the genome during embryo development. After fertilization, the oocyte and sperm genomes undergo major structural reorganization to ensure the establishment of **totipotency** and the transition to a **pluripotency** state and initial differentiations [54–57]. Genome reorganization includes the erasure of parental DNA methylation and *de novo* formation of heterochromatin, which are thought to be essential to ensure correct embryo development [54–56]. Remodeling is particularly evident at the paternal chromatin, which is subject to active DNA demethylation and nearly genome-wide replacement of protamines by histone variant H3.3 [58]. Centromeres also undergo drastic structural changes from the two-cell to the four-cell stages. Centromeres are organized around minor satellite repeats and flanked by major satellite repeats that constitute the pericentromeric heterochromatin, the structure of which is essential for chromosome segregation [59]. In mouse interphase somatic nuclei, pericentromeric and centromeric sequences of several chromosomes cluster together, forming chromocenters. By contrast, in the zygote, pericentromeric and **centromeric heterochromatin** surrounds most NPBs in a ring-like shape (Figure 2). The formation of pericentromeric rings follows asymmetric parental dynamics. In the maternal pronucleus, the pericentromeres form a ring on the surface of the NPB as early

as a few hours after pronuclear formation, whereas the positioning of the pericentromeric repeats around the male pronucleus occurs later [60]. The localization of pericentromeric repeats around NPBs occurs before the acquisition of their embryonic heterochromatin signature H3K27me3, suggesting that their spatial configuration is required for heterochromatic silencing [61]. Accordingly, tethering of the pericentromeric repeats from NPBs to the nuclear envelop caused defective heterochromatic silencing, impairment of chromocenter formation, and developmental arrest at the two- and eight-cell stages [61]. Interestingly, rRNA genes were found positioned only around the periphery of NPBs surrounded by pericentric heterochromatin, indicating that rRNA repeats are not automatically associated with NPBs [60].

After the zygotic stage, the embryonic genome undergoes further structural and functional changes. At the beginning of the second cell cycle, major satellites still associate with the NPBs, forming thick partial rims as in zygotes [60]. At the late two-cell stage, concomitantly with the 'major phase' of EGA, there is a burst in *de novo* major satellite transcription that rapidly decreases, becoming almost undetectable at the eight-cell stage [62]. The synthesis of pericentric transcripts was shown to be required for the reorganization of heterochromatin and further development [63]. Concomitant with this wave of transcription, pericentromeric heterochromatin associates with NPBs, forming spherical patches, which are indicative of chromatin compaction. At the four-cell stage, centric and pericentric heterochromatin form structures that resemble classical chromocenters. Finally, at the blastocyst stage, the overall nuclear organization becomes similar to that of somatic cell nuclei in terms of nucleolus structure and chromocenter organization [60,64].

Further evidence for a role of NPBs in the structural organization of heterochromatin came from studies using enucleolation, a method that allows microsurgical removal of NLBs and NPBs from either oocytes or zygotes [65,66]. Enucleolated oocytes are able to reinitiate meiosis and reach Metaphase II at the same rate as control oocytes. They can also be fertilized, and the resultant embryos form essentially normal pronuclei, although these do not contain NPBs [65], indicating that NLBs in the oocytes are required for the formation of NPBs in the zygote. Importantly, embryos generated with enucleolated oocytes failed to pass the first few cleavages and the replacement of nucleoli in oocytes with nucleoli from embryonic stem cells (ESCs) had a detrimental effect on embryo development, suggesting that NPBs of maternal origin are crucial for embryonic development. Remarkably, at the two-cell stage, embryos derived from enucleolated eggs showed similar rRNA production rates and pre-rRNA processing compared with control embryos, indicating that NPBs are not necessary for pre-rRNA synthesis in embryos [44]. The presence of NPBs in embryos appears to be critical in a defined time window, ~8–10-h post fertilization. The removal of NPBs 10 h after sperm injection did not affect embryo development and new nucleoli could form after several cell divisions, suggesting that the nucleoli can originate from *de novo* synthesized materials [67]. Data also suggest that the developmental defects observed in embryos fertilized with enucleolated oocytes are due to problems with chromosome segregation, mislocalization of centromeric proteins, abnormal first embryonic S-phase progression, and replication stress [44,68]. Consistent with defects linked to genome stability, during the first embryonic cell cycle, embryos lacking NPBs showed a significant reduction in major and minor satellite DNA and a decrease in satellite transcripts by more than half [44]. Interestingly, zygotes generated from enucleolated oocytes showed alterations in higher chromatin organization, as evidenced by the appearance of heterochromatin in the form of scattered dots [69]. Alterations in heterochromatin detected through DAPI staining were also reported in UBF-null embryos, which lack NPBs [48]. Studies indicated an important role of nucleoplasmin 2 (NPM2) in the function of NLB and NPB linked to genome organization during early embryo development [70]. In the absence of NPM2, oocytes and embryos lack NLB and NPB and show defects in

heterochromatin structures. Consistent with a role of NPM2 in nucleolus and heterochromatin, a recent study showed that the expression of NPM2 in *Npm2*-null oocytes sufficed to reconstitute nucleolar structures in enucleolated embryos, and rescued their first mitotic division and full-term development [68].

Taken together, these results support the idea that the nucleolus during early embryo development serves not only for the re-establishment of active somatic nucleoli and ribosome biogenesis, but might also provide a platform for the establishment of heterochromatic structures that are necessary for proper chromosome segregation and further development.

Nucleolus and rRNA Genes in Embryonic Stem Cells

In preimplantation embryos, the parental genomes reduce DNA methylation on a global scale with the exception of parental imprints [57,71,72]. The loss of global DNA methylation levels continues until the blastocyst stage, where the inner cell mass (ICM) is first specified, through downregulation of the DNA methylation machinery [72–75]. The pluripotency state in ICM can be immortalized *in vitro* through culturing of ESCs. Studies in ESCs revealed a chromatin state that is generally less condensed and largely devoid of compact heterochromatin blocks compared with lineage-committed cells. It is considered that this open and transcriptionally permissive state reflects the plasticity of the ESC genome to enter any distinct transcriptional programs for lineage specification [76,77]. The active state of the ESC genome also holds true for rRNA genes, which are all active in ESCs due to the lack of DNA methylation and repressive histone marks, such as H3K9me2 and H3K9me3 [23,78]. Accordingly, ESCs contain large nucleoli, indicating high ribosome biogenesis activity [79]. The formation of silent rRNA genes in ESCs is prevented through the impairment of NoRC recruitment to rRNA repeats, a process that implicates noncoding RNA-mediated mechanisms [23,24] (Figure 3). In ESCs, the critical step for the production of pRNA is impaired through inhibition of IGS-rRNA processing, which is mediated by DHX9. The association of TIP5 with the unprocessed IGS-rRNA prevents the interaction with TTF-1 bound to the promoter, thereby abrogating rRNA gene silencing. Acquisition of silent rRNA genes occurs only upon differentiation and co-occurs with the decrease in rRNA synthesis, indicating that the reduction in nucleolar transcription is an early event during differentiation [23,80,81]. Accordingly, IGS-rRNA processing and production of pRNA is reactivated only upon differentiation, thereby initiating pRNA-NoRC-mediated formation of silent rRNA copies [23]. Evidence indicates that the regulation of rRNA gene transcription and chromatin state in ESCs is a critical aspect of cell fate determination. rRNA gene transcription is downregulated upon differentiation of murine or human ESCs [23,78,80]. Constitutive expression of fibrillarin, which is indispensable for ribosome biogenesis, contributes to the maintenance of pluripotency states, whereas fibrillarin knockdown or treatment with the Pol I inhibitor actinomycin D, both inhibiting rRNA gene transcription, induce the expression of differentiation genes [81]. Furthermore, in human ESCs, the reduction in rRNA synthesis by the Pol I inhibitor CX-5461 induces the expression of markers for all three germ layers and reduces the expression of pluripotency markers [80]. Finally, the impairment of IGS-rRNA processing and TIP5 recruitment to rRNA genes in ESCs blocks the exit from pluripotency state [24].

Previous studies in somatic cells highlighted the nucleolus as one of the subnuclear compartments that serves for the organization of the inactive chromatin in the cell [4–8]. Recent data have also started to suggest that the nucleolus has an active role in the remodeling of the ESC genome, which, during differentiation, undergoes structural rearrangements and the formation of highly condensed and transcriptional repressed heterochromatic regions that cluster at the nucleolus or at the nuclear periphery [23,79,82–84]. Addition of mature pRNA into ESCs that contain only unprocessed IGS-rRNA was not only sufficient to establish rRNA gene silencing,

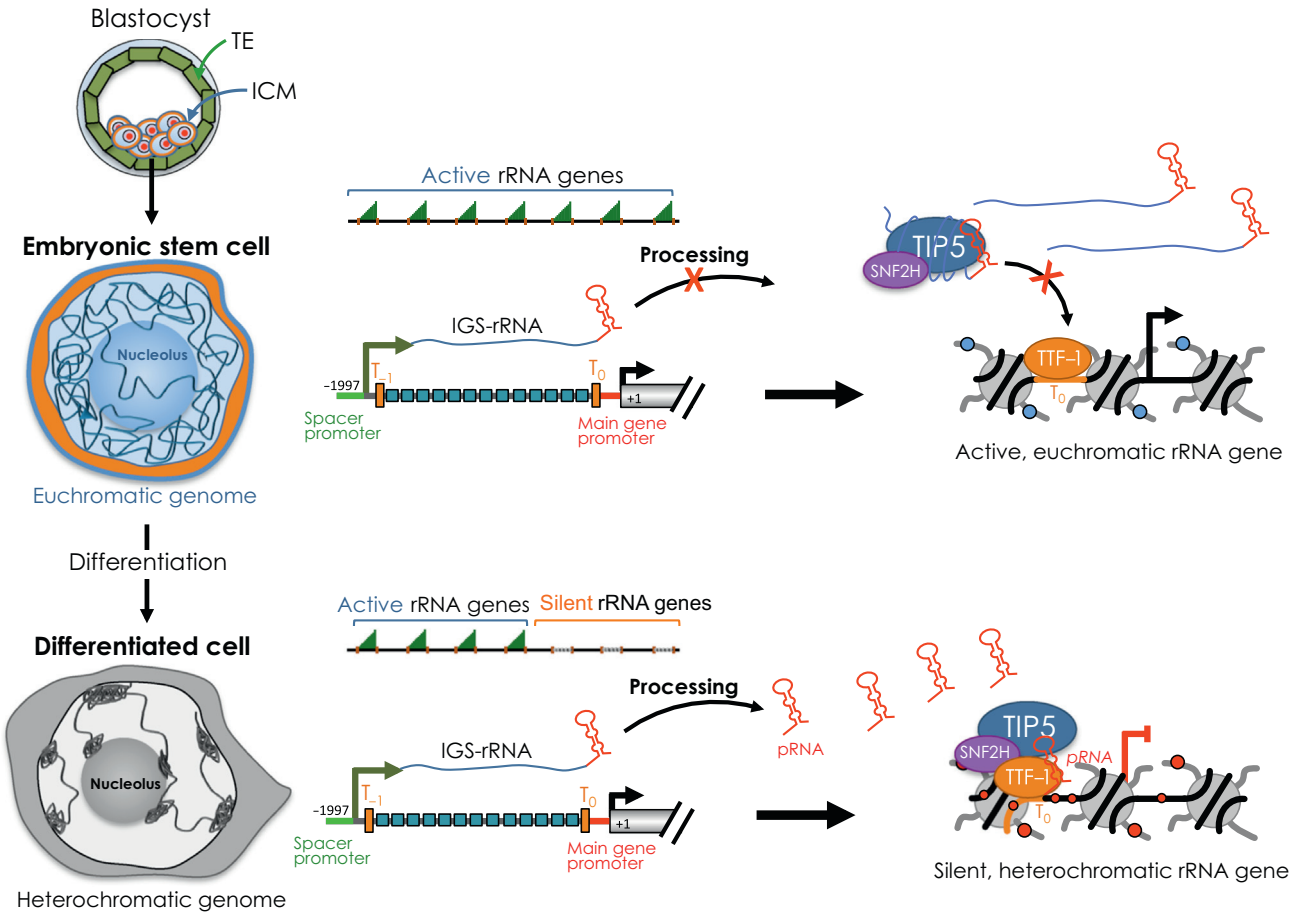

Figure 3. Establishment of Silent rRNA Genes during Embryonic Stem Cell (ESC) Differentiation. The pluripotency state in the inner cell mass (ICM) can be immortalized *in vitro* through culturing of ESCs. ESCs display an open and euchromatic genome that is remodeled upon differentiation into a condensed and heterochromatic state, including the formation of highly compact and transcriptionally repressed heterochromatic regions that cluster at the nucleolus or at the nuclear periphery. rRNA genes are all active in ESCs and establishment of silent copies occurs only upon differentiation [23,78]. In ESCs, the processing of intergenic spacer (IGS)-rRNA into pRNA is impaired. This process is reactivated only upon differentiation [23,24]. The association of TTF-1 interacting protein 5 (TIP5) with the unprocessed IGS-rRNA prevents the interaction with TTF-1, which is bound to the promoter, thereby abrogating rRNA gene silencing. Upon differentiation, processing of IGS-rRNA is activated allowing the production of mature pRNA, thereby promoting the TIP5–TTF1 interaction that is productive for guiding the nucleolar remodeling complex (NoRC) to rRNA genes and formation of heterochromatin at nucleoli. The formation of silent and heterochromatic rRNA genes co-occurs with the global remodeling of the genome from an euchromatic into a heterochromatic state, which might favor the exit from pluripotency. Abbreviation: TE, trophectoderm

but also induced the remodeling of the euchromatic ESC genome into a heterochromatic structure that is similar to that found in differentiated cells [23]. ESCs containing silent rRNA genes display highly condensed heterochromatic blocks outside the nucleolus, express genes implicated in cell differentiation, and lose their pluripotency state due to their inability to form teratoma. These results suggest that the formation of heterochromatin in the nucleolus at rRNA genes promotes the heterochromatinization of the rest of the nuclear genome and that the chromatin state of rRNA genes has an impact on genome organization. A similar observation was also made in NIH-3T3 mouse embryo fibroblast cells, which contain rRNA genes, half of which are in a heterochromatic state. The impairment of rRNA gene silencing through knockdown of TIP5 had an impact on pericentric heterochromatin, as evidenced by the decrease in repressive histone marks, such has H3K9me2 and H3K9me3 [85,86]. The link between rRNA genes and

genome architecture is also supported by results in *Drosophila* showing that the decrease in rRNA repeats globally reduces heterochromatin content in the genome and affects the expression of euchromatic genes [87,88]. Taken together, these results indicate that rRNA gene chromatin states affect nucleolus structure and are implicated in the regulation of genome architecture and cell fate decision.

Concluding Remarks and Future Perspectives

Increasing evidence indicates the nucleolus and rRNA genes as central players in genome architecture. Changes in nucleolus architecture and chromatin composition of rRNA genes, the genetic component of the nucleus, co-occur with the drastic genome reorganization during the critical phases of gametogenesis and early mammalian development. Although the exact mechanisms of this crosstalk remain to be elucidated, an attractive hypothesis is that changes in the transcription and chromatin state of rRNA genes affects the nucleolus compartment in its structure and protein composition, allowing the concentration of factors required for the establishment of repressive states according to developmental cell stage (see Outstanding Questions). This is particularly evident during ESC differentiation, where targeting of silencing in the nucleolus at rRNA genes induced heterochromatin formation in the rest of genome. Future research will be needed to improve methods to define how and which genomic regions associate with the nucleolus according to cell state, and to determine the molecular mechanisms of how the organization of the genome around the nucleolus is established, its dynamics, and its functional role during early development and in disease.

Outstanding Questions

How are the chromatin and epigenetic features of rRNA genes regulated during gametogenesis and early embryo development?

Which mechanisms drive the formation of heterochromatin ring-like structures around the NLB of SN oocytes and the NPB in the pronuclei of zygotes? What is the function of these structures?

Which mechanisms regulate the crosstalk between the nucleolus and repressive chromatin domains? How does the nucleolus affect genome architecture? How do genomic regions contact the nucleolus upon ESC differentiation?

What is the temporal order of chromatin changes during ESC differentiation? Are some changes necessary for others to occur?

Is there a specific class of rRNA genes that is preferentially converted into silent copies during ESC differentiation?

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